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Calcitriol reduces the occurrence of acute cellular rejection of liver transplants: a prospective controlled study

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Objective: To investigate the acute cellular rejection rate of liver transplant recipients taking or not taking calcitriol in a prospective, randomized, controlled clinical study. *Methods:* Primary liver transplant patients were randomized to receive the placebo (arm A), calcium gluconate (arm B) or calcium gluconate plus calcitriol (arm C). The composition of circulating T cell subpopulations was determined by flow cytometry at baseline and one week post transplant. The primary endpoint was acute cellular rejection rate at one month post transplant. *Results:* Seventy-five liver transplant recipients were eligible, including 25 patients each in arm A, B and arm C. The mean baseline serum 25-(OH) vitamin D content was 12.5 ± 3.0 (range, 2.5 to 19.5) ng/mL, with no statistical difference among the three arms. Among 7 (9.33%) patients who developed definite acute cellular rejection (Banff score ≥ 6), only 1 (4%, 1/25) were from arm C while 6 (12%, 6/50) of them were from either arm A or B. The acute cellular rejection rate was 20% (5/25) for arm C, 32% (8/25) for arm A and 28% (7/25) for arm B (arm C vs. arm A or B, $P < 0.05$). Flow cytometric analysis showed that the proportion of circulating $CD4^+$ $CD25^{high}$ CD^{127-}(Treg) cells in arm C increased by 63.22% at one week post transplant (3.95 ± 4.0 %) compared to baseline (2.42 ± 2.35 %) ($P < 0.05$). No significant difference was observed in arm A or B ($P > 0.05$ in both vs. baseline). Furthermore, no significant difference was noted among the three arms in the proportion of $CD4^+CD45RA^-CD45RO^+$, $CD4^+CD25^{low}CD45RA^+$ and $CD4^+CD25^{low}CD45RA^-$ T cells at one week post transplant compared to baseline. *Conclusion:* Calcitriol has apparent beneficial effect on acute cellular rejection of liver transplants, which may be associated with increases in the proportion of circulating Treg cells.

1. Introduction

Acute cellular rejection remains a common problem following liver transplantation (Margarit et al. 1998) and the one-year acute cellular rejection rate is 30 to 70%, and most of them occur in the first month after transplantation (Fisher et al. 1995). Although simple acute cellular rejection may herald the development of chronic rejection in certain liver transplant recipients, it generally does not appear to affect the long term function of a transplant (Demetris et al. 2002; Dousset et al. 1998; Sánchez-Fueyo and Strom 2011). Calcineurin inhibitors could effectively prevent acute cellular rejection, but serious dose-dependent side effects, particularly kidney injury, diabetes, hypertension, neurotoxicity and hyperlipidemia, hamper their use (Geissler and Schlitt 2009). Therefore, calcineurin inhibitors are combined with other immunosuppressive agents such as mycophenolate mofetil or sirolimus to achieve immune suppression. However, mycophenolate mofetil and sirolimus cause apparent bone marrow suppression; additionally, mycophenolate mofetil increases the chance of infection in transplant recipients (Farkas et al. 2009).

Calcitriol is a classic medication for the treatment of osteoporosis and has been shown to reduce acute cellular rejection rate after renal transplant (Tanaci et al. 2003), and heart transplant (Briffa et al. 2003; Shane et al. 2004). Calcitriol is used by liver transplant recipients for immunosuppressive drug-induced osteoporosis (Wagner et al. 2012). Accumulating evidence indicates that calcitriol also possesses immunomodulatory function (Bitetto et al. 2010), but there have been few studies on the outcome of liver transplant recipients receiving calcitriol and its correlation with immunologic changes.

Here, we investigated the acute cellular rejection rate of liver transplant recipients taking or not taking calcitriol in a prospective, randomized, controlled clinical study.

2. Investigations and results

2.1. Patient demographic and baseline characteristics

Seventy-five patients undergoing liver transplantation were eligible for the study. Patient demographic and baseline characteristics are shown in Table 1. Most of them (82.7%) were male and the median age of the patients was 48.5 (range, 28 to 65) years. The mean MELD score of these patients were 13.8 (range 6 to 35). The most common cause of liver transplantation was hepatic B virus-associated liver cirrhosis (82.7%). The majority

Table 1: Demographic and baseline characteristics of transplant recipients

Variable	Total N = 75	Arm A N = 25	Arm B N = 25	Arm C N = 25
Male gender	62 (82.7%)	21	22	19
Age, years	48.5 (28–65)	48.7	48.1	49.0
> 55	20	6	6	8
Body mass index, kg/m^2	20.7 (14.3–35.5)	19.8	20.46	22.06
< 25	47	17	16	14
MELD score	13.8 (6–35)	14.7	13.1	12.6
> 15	24	9	8	7
Recipient diagnosis				
HBV	62 (82.7%)	19	22	21
Alcoholic liver cirrhosis	4 (5.3%)	3	1	0
Autoimmune liver disease	9 (12%)	3	2	4
Pretransplant diabetes	9 (12%)	2	4	3
Immunosuppressive regimen				
Tacrolimus plus MMF	66 (78%)	22	25	19
Cyclosporine plus MMF	5 (20.6%)	2	0	3
Sirolimus or others	4 (1.5%)	1	0	3
Serum 25(OH) vitamin D, ng/mL		12.5 ± 3.0	12.5 ± 3.0	12.5 ± 3.0
≤ 5.0	7	4	3	0
> 5 or ≤ 12.5	31	17	13	1
> 12.5	37	4	9	24

CG, calcium gluconate; HBV, hepatitis B virus; MELD, Model of End-Stage Liver Disease; MMF, mycophenolate mofetil.

of the patients (78%) received tacrolimus (plus mycophenolate mofetil) for immunosuppression followed by cyclosporine (plus mycophenolate mofetil) (20.6%). 25 patients received calcium gluconate plus calcitriol (arm C) while other 50 patients received calcium gluconate (arm B) or the placebo (arm A). The three arms were generally well balanced in demographic and baseline characteristics.

2.2. Serum 25-(OH) vitamin D content of the study subjects

The mean serum 25-(OH) vitamin D content in the study cohort at baseline was 12.5 ± 3.0 (range 2.5 to 19.5) ng/mL. Approximately half of the patients (49.33%) had a serum 25-(OH) vitamin D content greater than 12.5 ng/mL while 41.33% of the patients had a serum 25-(OH) vitamin D content between 5 and 12.5 ng/mL. Twelve (60%, 12/20) patients aged more than 55 years, 15 (62.50%, 15/24) patients with a MELD score > 15, and 23 (48.94%, 23/47) patients with a body mass index < 25 kg/m^2 had a serum 25-(OH) vitamin D content between 5 and 12.5 ng/mL (Table 2). The mean serum 25-(OH) vitamin D content at baseline was comparable among the control arm, the calcium gluconate arm and the calcium gluconate plus calcitriol arm ($P < 0.05$ in all).

2.3. Calcitriol reduced the rate of acute cellular rejection in liver transplant recipients

We evaluated the development of acute cellular rejection in the study cohort using the Banff schema. Seven (9.33%) patients developed definite acute cellular rejection (Banff score ≥6). Only 1 (4%, 1/20) of the patients who developed acute cellular rejection were from arm C while 6 (12%, 6/50) of these patients were from either arm A (n = 25) or B (n = 25). The acute cellular rejection rate was 20% (5/25) for arm C, 32% (8/25) for arm A and 28% (7/25) for arm B (arm C vs. arm A or B, $P < 0.05$), suggesting that calcitriol markedly reduced the rate of acute cellular rejection in liver transplant patients. On the other hand, age > 55 years, body mass index, MELD score, or pretransplant diabetes had no impact on the rate of acute cellular rejection (Table 3). In addition, 7 (35%, 7/20) of those patients who developed acute cellular rejection had moderate to severe acute cellular rejection with no statistically significant difference in their rate among arm A, B and C ($P < 0.5$) (Table 4). However, Spearman correlation analyses suggested that development of moderate to severe acute cellular rejection correlated with pretransplant 25-(OH) vitamin D levels. Three (42.86%, 3/7) out of the 7 patients whose serum 25-(OH) vitamin D content was < 5.0 ng/mL developed moderate to severe acute cellular rejection, which, however, was seen in only 2 out of 35 patients (5.71%) whose serum 25-(OH)

Table 2: Serum 25(OH) vitamin D contents in liver transplant recipients at baseline

	≤5.0 ng/mL	> 5/≤12.5 ng/mL	> 12.5 ng/mL
All recipients (n = 75)	7	31	37
Age > 55 years	4	12	4
Body mass index < 25 kg/m^2	5	23	19
MELD score > 15	4	15	5
Pretransplant diabetes	2	4	3
Arm A	4	17	4
Arm B	3	13	9
Arm C	0	1	24

MELD, Model of End-Stage Liver Disease.

Table 3: Development of moderate to severe acute cellular rejection (Banff score ≥6) in liver transplant recipients in one month post transplantation

Variable	Banff score < 6 n = 68	Banff score ≥6 n = 7	*P*
Age > 55 years	18	2	
Female recipients	13	2	
Body mass index < 25 kg/m^2	26	2	
MELD score > 15	9	0	
Pretransplant diabetes	21	3	
Pretransplant 25(OH) vitamin D, ng/mL			
≤5.0	4	3	
> 5 or ≤12.5	29	2	
> 12.5	35	2	< 0.05
Arm A	23	2	
Arm B	21	4	
Arm C	24	1	

MELD, Model of End-Stage Liver Disease.

vitamin D content was < 12.5. ng/mL or of 29 patients (6.90%) whose serum 25-(OH) vitamin D content was between 5 and 12.5. ng/mL.

2.4. Calcitriol increases the proportion of Tregs cells in the peripheral blood of liver transplant recipients

Our flow cytometric analysis showed that the proportion of $CD4^+$ $CD25^{high}$ CD^{127-} (Treg) cells in the peripheral blood of liver transplant recipients in arm C increased by 63.22% at one week post transplant ($3.95 \pm 4.0\%$) compared to that before transplant ($2.42 \pm 2.35\%$) ($P < 0.05$) while no significant difference was observed in arm A or B ($P > 0.05$ in both) (Fig. 1a). Furthermore, no significant difference was noted among the three arms in the proportion of $CD4^+CD45RA^-CD45RO^+$ (human IL-17-producing T cells), $CD4^+CD25^{low}CD45RA^+$ (naïve T cells) and $CD4^+CD25^{low}CD45RA^-$ (memory T cells) at one week post transplant compared to baseline (Fig. 1c to 1e).

2.5. Calcitriol induces distinct changes in cytokine profile in the peripheral blood of liver transplant recipients

Serum IL-2 content markedly increased in all three arms at one week post transplant compared to baseline ($P < 0.05$ in all vs. baseline); however, this increase was significantly less in arm C compared to arm A or B ($P < 0.05$) (Fig. 2a). Serum IL-4 contents mildly decreased over time across all three arms ($P > 0.05$ in all vs. baseline), but no noticeable difference among the three arms at week 1 or 4 post transplant ($P > 0.05$) (Fig. 2b). Serum IL-6, and IL-10 content markedly increased in all three arms at one week post transplant compared to baseline ($P < 0.05$ in all vs. baseline); however, this increase was significantly less in arm C compared to arm A or B ($P < 0.05$) (Fig. 2c and 2e). Additionally, serum GM-CSF contents were markedly increased in arm A and B at one week post transplant compared to baseline ($P < 0.05$ in both vs. baseline) while serum GM-CSF contents were noticeably lower in arm C than those of arm A or B ($P < 0.05$) at 1 week post transplant.

Table 4: Development of definite acute cellular rejection (Banff score≥3) in liver transplant recipients beyond one month post transplantation

Variable	Banff score < 3 n = 55	Banff score ≥3 n = 20	*P*
Age > 55 years	17	3	
Female recipients	11	4	
Body mass index < 25 kg/m^2	19	9	
Pretransplant diabetes	7	2	
MELD score > 15	16	8	
Arm A, n(%)	17	8	
Arm B, n(%)	18	7	
Arm C, n(%)	20	5	< 0.05

MELD, Model of End-Stage Liver Disease.

3. Discussion

In the current study, we demonstrated that calcitriol supplementation was associated with a marked reduction in the rate of acute cellular rejection of liver transplants at one month post transplant. Acute cellular rejection was observed in only 10% (5/50) of transplant recipients taking calcitriol but in 80% (16/20) of transplant recipients not taking the drug, suggesting an apparent beneficial effect of calcitriol on acute cellular rejection. We also observed a lower rate of graft rejection in liver transplant recipients at one year post transplantation (unpublished data). Vitamin D undergoes hydroxylation in the liver to become 25-(OH) vitamin D and a second hydroxylation in the kidney to become activated, forming 1,25-(OH) vitamin D. Upon entry into cells, 1,25-(OH) vitamin D binds to the vitamin D receptor to activate gene transcription. Apart from its regulatory role in calcium metabolism, vitamin D possesses immunomodulatory effects, directly on antigen presenting cells and activated T cells or by modulating the gene activities in producing cytokines. The apparent beneficial effect of calcitriol on acute cellular rejection may be explained by the concept of "window of opportunity of immunological engagement" (Dresske et al. 1998). Calne and Dresske (Calne 2004) postulated that there was a window of opportunity for immunological engagement in the early post transplant period during which immunological interaction between donor and recipient immunogenicity may allow for the development of tolerance, consequently leading to improved graft survival.

Certain patients can maintain normal liver function with a lack of acute or chronic rejection after complete withdrawal of immunosuppressive drugs, a phenomenon termed operational tolerance, while lack of graft rejection when the doses of immunosuppressive drugs are markedly reduced is considered minimal immunosuppressive tolerance. Development of tolerance may be associated with changes in the proportion of Treg cells. Sakaguchi et al.(2005) showed that Tregs cells are derived from $CD4^+CD25^+$ cells of thymic origin and account for 5 to 10% of

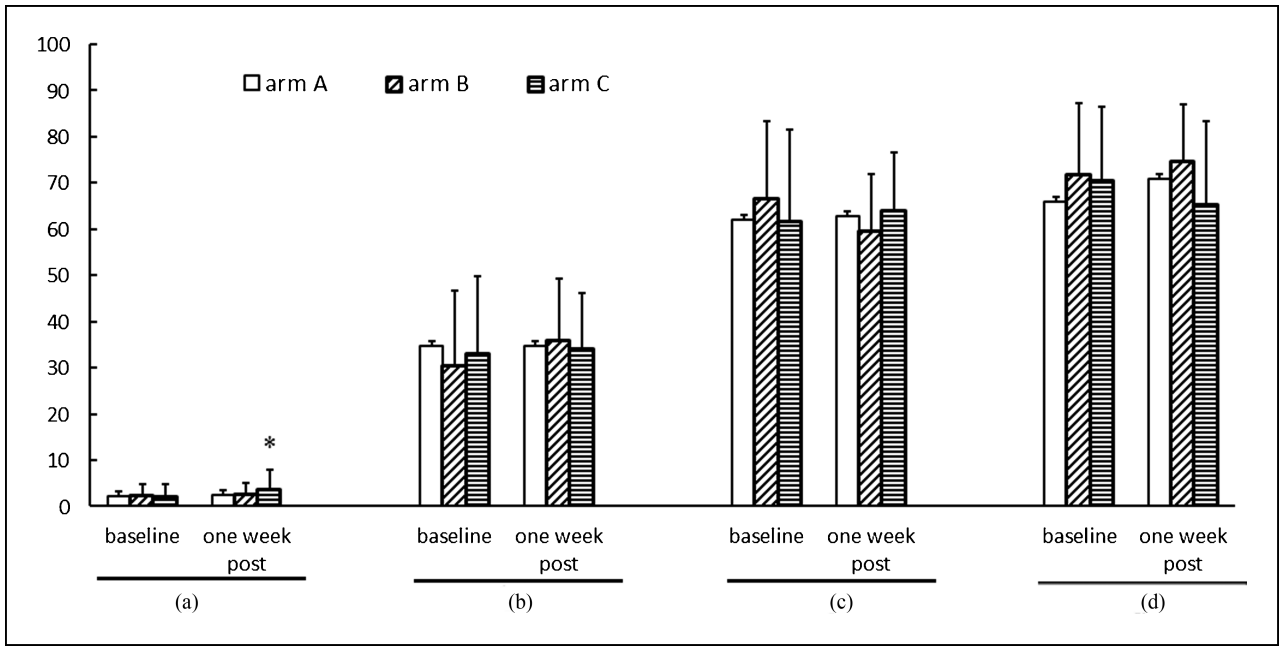

Fig. 1: Flow cytometric analysis of the composition of circulating T lymphocyte subpopulations at baseline and one weeks post transplant. (a) Circulating $CD4^+$ $CD25^{high}$ CD^{127-} (Treg) cells; (b) $CD4^+CD45RA^-CD45RO^+$ (human IL-17-producing T cells), (c) $CD4^+CD25^{low}CD45RA^+$ (naïve T cells) and (d) $CD4^+CD25^{low}CD45RA^-$ (memory T cells). Data shown are mean(sd) of at least three independent experiments. $^*p < 0.05$ vs baseline.

lymphocytes in the peripheral blood. The number of Treg cells increases in kidney transplant recipients (Mantel et al. 2006). It has been found that Treg cells are involved in preventing against graft rejection and inducing immunologic tolerance (Wieërs et al. 2007). Our flow cytometric analysis showed that, compared to baseline, the proportion of Treg cells in the peripheral blood of liver transplant recipients taking calcitriol increased by 63.22% at one week post transplant while no noticeable increase was observed in transplant recipients not taking the study medication. Our Spearman analysis further indicated that reduction in the rate of acute cellular rejection correlated with increase in the percentage of circulating Treg cells.

Our study has several limitations. The study was performed in a single institution and included only liver transplant recipients, and therefore the results may not apply to other centers or other types of organ transplantation. In addition, the number of subjects in the current study is limited. Furthermore, we only investigated the one-month outcome of liver grafts. While acute cellular rejection may herald the development of chronic rejection in certain liver graft recipients, there is no evidence that acute cellular rejection is associated with a worse long-term outcome. These issues need to be addressed in clinical trials that involve a larger patient populaiton with longer follow up.

In summary, we have demonstrated that calcitriol has apparent beneficial effects on acute cellular rejection of liver transplants. This may be associated with increases in the proportion of circulating Treg cells. Our study provides the first clinical evidence that calcitriol possesses immunomodulatory activities apart from its regulatory effect on bone metabolism.

4. Experimental

4.1. Patients

We prospectively recruited liver transplant patients between March 1, 2010 and March 31, 2011 at a large-volume transplant center of Shanghai First People's Hospital. A patient was included in the study if he or she fulfilled the following requirements: 1) the patient received a primary liver transplant, 2) the patient received primary immunosuppression with tacrolimus or cyclosporine A or other immunosuppressive agents, and 3) the patient survived for more than 5 days following liver transplantation without graft failure. A patient was excluded from the study if he or she received treatment for suspected rejection in the absence of liver biopsy or not confirmed by histology.

The study protocol was approved by the local institutional review board at the authors' affiliated institution and informed written consent was obtained from the patients or their legal surrogates. The study was carried out following good clinical practice and in accordance with the Declaration of Helsinki.

4.2. Study intervention

Patients were randomized at an allocation ratio of 1:1:1 to receive the placebo (arm A), calcium gluconate (arm B) or calcium gluconate plus calcitriol (arm C). The placebo was identical to calcitriol in appearance and color. No effective ingredients were found in the placebo by drug testing. Calcium gluconate (10%, 20 mL) was given intravenously daily in arm B and C, and calcitriol was given orally at 0.25 μg daily in arm C.

4.3. Clinical evaluation

Demographics, including age and gender, were collected for the recipients. The laboratory values for creatinine, total bilirubin, and the international normalized ratio (INR) immediately preceding transplantation were used to calculate the Model for End-Stage Liver Disease (MELD) score. Calcitriol levels were determined by chemiluminescence immunoassays using commercially available kits and 25-OH vitamin D levels were measured using automatic chemiluminescence immunoassay analyzer LIAISON.

4.4. Immunosuppressive drugs regimen

All patients received 500 mg methylprednisolone for one week intraoperatively. The postoperative immunosuppressive regimen included tacrolimus or cyclosporine A plus mycophenolate mofetil. Tacrolimus trough concentration was set at 5–10 ng/mL within one month after transplantation, and cyclosporine A concentration was set at 800–1200 ng/mL 2 h after administration. Moderate to severe acute cellular rejection patients were pulsed with methylprednisolone at 300 mg/day given four times intravenously for 3 days continuously and thereafter at 240 mg/day and thereafter tapered to 40 mg at 40 mg/day when the patients were switched to oral prednisone 20 mg/day.

4.5. Flow cytometry

Immunomonitoring was performed before transplantation and one week after transplantation on blood samples from all participating patients to assess the composition of circulating T cell subpopulations. Briefly, mononuclear cells were isolated from 10 mL heparinized peripheral blood followed by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. Lymphocyte immunophenotyping for the following antigens, CD3, CD4, CD25, CD127, CD45RA, and CD45RO was performed by flow cytometry using the tri-color fluorescent direct labeling

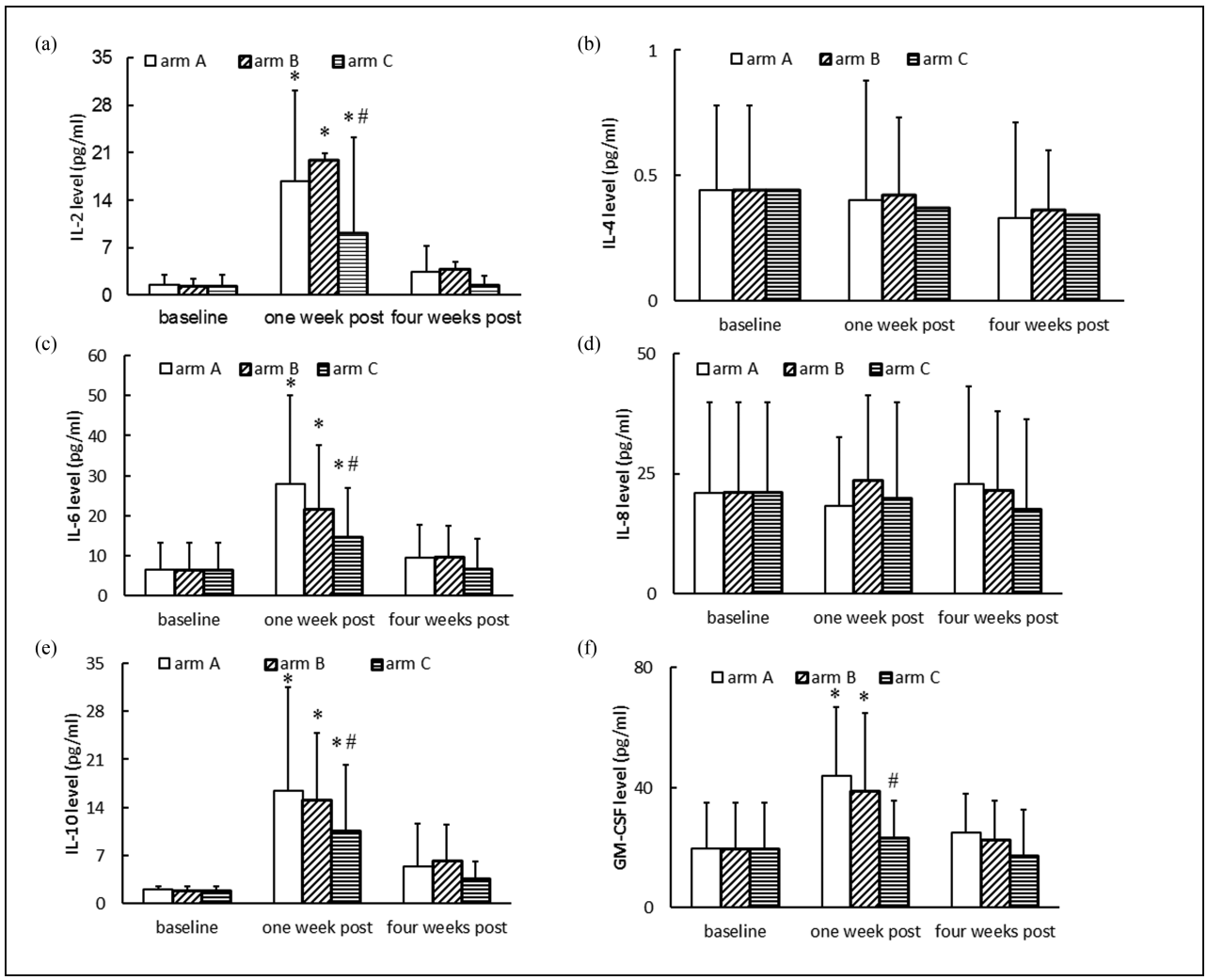

Fig. 2: Cytokine profiles of liver transplant recipients at baseline and 1 and 4 weeks post transplant. Serum contents of (a) IL-2; (b) IL-4; (c) IL-6, (d) IL-8, (e) IL-10 and (f) GM-CSF were determined by. using the Bio-Plex suspension array system. Data shown are mean (s.d.) of at least three independent experiments. *$p < 0.05$ vs baseline; #$p < 0.05$ vs arm A or B at one week post transplant.

method. Briefly, 1 mL heparinized peripheral blood was collected one week before and one and one week after liver transplantation. All antibodies were purchased from BD Biotech (New Jersey, CA). Data were acquired with logarithmic sampling using the Beckman Coulter Epics XL flow cytometer and CellQuest software. CD45/side scatter-based gating was used to delineate lymphocytes, and CD45/CD3-based gating was used to delineate T cell (Voo et al. 2009).

4.6. Enzyme-linked immunosorbent assays (ELISA)

Cytokine levels in peripheral blood were quantified in duplicate using the Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol (Liu et al. 2009). The following cytokines were measured simultaneously: IL-2, IL-4, IL-6, IL-8, IL-10, and GM-CSF.

4.7. Grading of acute cellular rejection

Pathologic diagnosis was made by two similarly experienced liver transplant pathologists and scores were averaged for the Banff rejection activity index (Demetris, 1995; Rejection 1997) (RAI). A RAI score < 2 were classified as no rejection, 2 as indeterminate rejection, 3 to 5 as mild rejection, and ≥ 6 as moderate or severe rejection.

4.8. Statistical analysis

The statistical analyses were pre-specified and performed on an intention-to-treat basis with the inclusion of all patients who underwent randomization. Both full and per-protocol analysis were used. The full analysis sets included all patients who were randomized to treatment and had a baseline assessment and at least one post-baseline assessment. The per-protocol sets included all evaluable patients who completed calcitriol treatment and were not excluded as protocol violators. Unless otherwise specified, all efficacy results reported herein were based on the full analysis wherein, for patients who withdrew or were lost to follow-up, we used the last observation carried forward approach.

The primary study endpoint was acute cellular rejection rate at one month post transplant. The secondary study endpoints included changes from baseline in composition of circulating T lymphocyte subpopulations and changes in cytokine profiles at 4 weeks post transplant.

Data were expressed as mean standard deviation (s.d.) and analyzed with the statistical software SPSS version 11.0 (SPSS Inc., Chicago, IL). F analysis was used for comparison among multiple arms and two side Student's t test was used for comparison between arms. Spearman correlation analyses were performed to determine correlation between variables. All analyses were performed using a level of significance at 5%.

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